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Review

Lipoxygenases – Structure and reaction mechanism

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ABSTRACT

Lipid oxidation is a common metabolic reaction in all biological systems, appearing in developmentally regulated processes and as response to abiotic and biotic stresses. Products derived from lipid oxidation processes are collectively named oxylipins. Initial lipid oxidation may either occur by chemical reactions or is derived from the action of enzymes. In plants this reaction is mainly catalyzed by lipoxygenase (LOXs) enzymes and during recent years analysis of different plant LOXs revealed insights into their enzyme mechanism. This review aims at giving an overview of concepts explaining the catalytic mechanism of LOXs as well as the different regio- and stereo-specificities of these enzymes.

Contents

1. Introduction

Fatty acids, major building blocks of lipids, are major components of membranes and storage lipids. Apart from the turnover of fatty acids within lipids and their use as carbon and energy source, oxidation of unsaturated fatty acids is a major reaction in lipid metabolism (Feussner and Wasternack, 2002). The formation of fatty acid hydroperoxides may occur either by chemical oxidation or by the action of enzymes such as lipoxygenase (LOX) (Andreou et al., 2009b; Mosblech et al., 2009). The metabolites that derive from oxidation of polyunsaturated fatty acids (PUFAs) via a LOX-catalyzed step as well as those metabolites that derive from alternative oxidation reactions and subsequent reactions are collectively named oxylipins and the metabolic pathways involved in oxylipin formation are collectively called oxylipin pathway. In a number of reviews the diversity of oxylipins, the enzymes involved in their formation as well as their physiological function in non-mammals has been summarized (i.e., Andreou et al., 2009b; Blée, 2002; Gerwick et al., 1999; Howe and Jander, 2008; Liavonchanka and Feussner, 2006; Mosblech et al., 2009; Oliw, 2002; Pohnert, 2005; Schneider et al., 2007b; Stumpe and Feussner, 2006; Tsitsigiannis and Keller, 2007; Wasternack, 2007). Recent genome sequences shed new light on the structure and reaction mechanism of LOXs. Consequently, our knowledge about LOXs in an increasing number of organisms has been substantiated and new aspects are reviewed here.

2. LOX enzymes

LOXs are a family of non-heme iron containing dioxygenases (Brash, 1999; Schneider et al., 2007b). They catalyze the insertion

Abbreviations: HPODE, hydroperoxy octadecadienoic acid; LA, linoleic acid; LOX, lipoxygenase; PUFA, polyunsaturated fatty acid.

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of molecular oxygen into PUFAs that contain one or more (1*Z*,4*Z*)-pentadiene moieties to yield the corresponding (1*S*,2*E*,4*Z*)-hydroperoxides (Liavonchanka and Feussner, 2006). LOXs occur ubiquitously in plants and mammals, and only recently they have been detected in coral, moss, fungi and a number of bacteria as well (Andreou et al., 2009b; Oliw, 2002). LOX-derived fatty acid hydroperoxides can be further metabolized into volatile aldehydes and jasmonates in plants (Mosblech et al., 2009), in diols and lactones in fungi (Tsitsigiannis and Keller, 2007) and in lipoxins and leukotrienes in mammals (Samuelsson et al., 1987; Sigal et al., 1994). These molecules play an important role as signals in wound healing and defense processes in plants, while in mammals they are involved in inflammation, asthma and heart disease. In fungi, they play a role in regulation of mycotoxin production and of the sexual and asexual life cycle (Tsitsigiannis and Keller, 2007). Nothing is known till now about the biological function of these enzymes in prokaryotes.

Plant LOXs are monomeric proteins of about 95–100 kDa that consist of two domains. The amino-terminal domain of about 25–30 kDa is a β-barrel domain that is structurally related to so-called C2 domains (Corbin et al., 2007). Its exact function is yet unknown, but an involvement in membrane or substrate binding has been discussed (May et al., 2000; Tatulian et al., 1998). The carboxy-terminal domain of about 55–65 kDa consists primarily of α-helices and harbours the catalytic site of the enzyme (Schneider et al., 2007b). LOX enzymes contain one iron atom per protein molecule. In addition, LOXs have been described originating from fungi that harbour a manganese atom instead, in the active site (Andreou et al., 2009b; Oliw, 2002). The iron active site metal is a non-heme iron that is octaedrically coordinated by 5 five amino acid side chains and a water or hydroxide ligand (Fig. 1). In case of plant LOXs these residues are always three histidines, one asparagine and the carboxy group of the carboxy-terminal isoleucine. In mammalian LOXs, however, the iron is coordinated by four histidines and again the carboxy-terminal isoleucine. LOXs are versatile catalysts, because they are multifunctional enzymes, catalyzing at least three different reactions: (i) oxygenation of substrates (dioxygenase reaction), (ii) secondary conversion of hydroperoxy lipids (hydroperoxidase reaction), and (iii) formation of epoxy leukotrienes (leukotriene synthase reaction). However, under physiological conditions the first reaction is most prevalent in plants (Liavonchanka and Feussner, 2006).

2.1. Regio-specificity

For years LOXs have been classified according to their pH optimum. Those having an alkaline pH optimum were designated as type 1-LOXs, while type 2-LOX isoforms have a pH optimum around neutral pH (Siedow, 1991). During recent years, however, plant LOXs are classified in two alternative ways: (i) according to

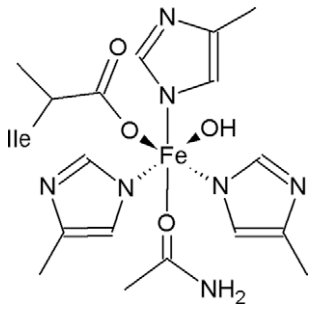

Fig. 1. Active site coordination sphere of the plant LOXs. The figure shows the active site of soybean LOX2 and was adapted from http://metallo.scripps.edu/PROMISE/2SBL.html.

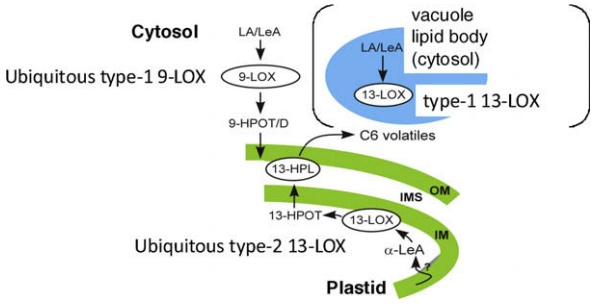

Fig. 2. Intracellular location of LOX pathway reactions. OM/IM, outer and inner membrane of the chloroplast envelope; IMS, inter membrane space. Scheme modified according to Froehlich et al. (2001).

their subcellular localization: extraplastidial enzymes harbouring no transit peptide and having a high sequence similarity (>75%) to one another. They are designated again as type 1-LOXs. (ii) Plastidial LOXs that carry a chloroplast transit peptide sequence. These enzymes show only a moderate overall sequence similarity (>35%) to one another and have been classified as type 2-LOXs (Shibata et al., 1994). These latter LOX forms all belong to date to the subfamily of 13-LOXs (Fig. 2). In addition, it should be stressed that in case of type 1-LOXs only 9-LOXs have been ubiquitously found so far.

A second way to classify plant LOXs is with respect to their positional specificity of fatty acid oxygenation against linoleic acid (LA). With LA as substrate molecular oxygen can be introduced either at carbon atom 9 (9-LOX) or at carbon atom 13 (13-LOX) of the hydrocarbon backbone, which leads to the formation of 9-hydroperoxy and 13-hydroperoxy derivatives of LA (9- and 13-HPODE), respectively (Liavonchanka and Feussner, 2006) (Fig. 3). The two different regioisomers of hydroperoxy fatty acids may result from two independent properties of catalysis (Hamberg and Samuelsson, 1967). (i) Stereoselective hydrogen removal. For plants a number of studies have shown that in C-18 PUFAs this is almost only possible at C-11 (Feussner and Kühn, 2000). (ii) Selectivity of the site of antarafacial oxygen insertion via rearrangement of the intermediate fatty acid radical. Consequently, when hydrogen is abstracted at C-11, molecular oxygen can be introduced at position [+2] or [−2] leading to dioxygen insertion at C-13 or C-9. In mammals, LOXs are similarly classified according to their positional specificity of arachidonic acid oxygenation, which can take place either at positions C-5 (5-LOX), C-8 (8-LOX), C-9 (9-LOX), C-11 (11-LOX), C-12 (12-LOX) or C-15 (15-LOX) (Schneider et al., 2007b). Besides the high regio-specificity, the insertion of oxygen exhibits also high stereo-specificity dependent on the primary sequence of the enzyme, which is predicted to determine the orientation and depth of substrate penetration into the active site (Feussner and Kühn, 2000; Schneider et al., 2007b). However, it should be stressed that until now there is no unifying concept explaining the structural basis for the regio-specificity of all plant LOXs. This is even the case for LOXs having no regio- or a dual-specificity. They belong mainly to the group of legume and solanaceous cytosolic 13-LOXs (i.e., Hughes et al., 2001a,b). In addition, there exist LOXs that show high product specificity against C-18 PUFAs while they have no or a different specificity against C-20 PUFAs and vice versa (Andreou et al., 2009a; Feussner and Kühn, 1995).

Over the last decade a number of critical amino acids have been identified at the active site of selected LOX isozymes, which influence the regio-specificity of this group of enzymes (Liavonchanka and Feussner, 2006; Schneider et al., 2007b). Moreover, the so-called substrate orientation hypothesis may be the preferred model to explain the regio-specificity for plant LOXs, since hydrogen is only abstracted at C-11 in case of all available C-18 substrates

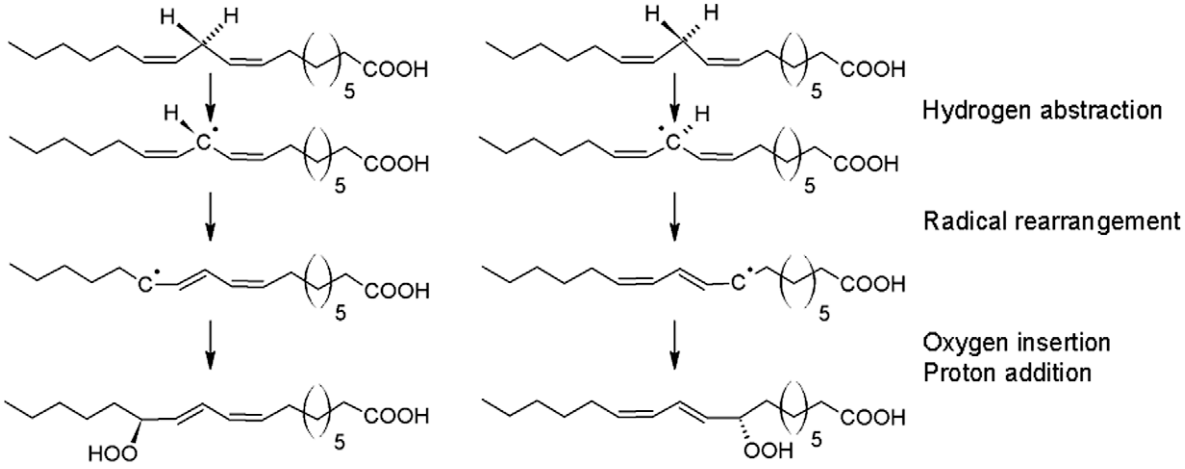

Fig. 3. The LOX reaction and the regio-specificity of the reaction mechanism.

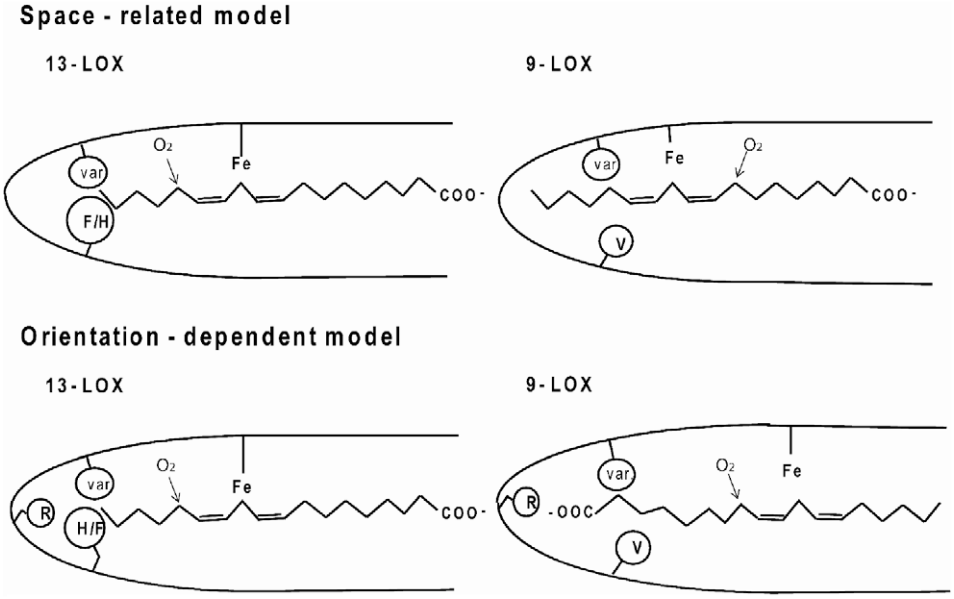

Fig. 4. Comparison of the two different models of substrate alignment explaining the positional specificity of LOXs. Space related model: straight substrate alignment at the active site of LOXs for both positional isomers. Orientation-dependent model: straight- and inverse-substrate orientation at the active site of LOXs for the two different positional isomers.

Table 1
Alignment of amino acid residues determining positional specificity in plant LOXs.

Enzyme	Accession number	Position of amino acid residues	Amino acid residues
9-LOX			
Solanum tuberosum LOX	P37831	575/576	Thr/Val
Arabidopsis thaliana LOX1	Q06327	576/577	Thr/Val
Hordeum vulgare LOXA	L35931	574/575	Thr/Val
Cucumis sativus LOX	CAB83038	594/595	Thr/Val
Solanum lycopersicum LOXA	AAA53184	578/579	Thr/Val
13-LOX			
Glycine max LOX1	P08170	556/557	Thr/Phe
Oryza sativa LOX1	BAA03102	678/679	Ser/Phe
Arabidopsis thaliana LOX4	CAC1964	642/643	Cys/Phe
Physcomitrella patens LOX	CAE47464	654/655	His/Phe
Mormordica charantia	AM930395	598/599	Thr/Gln
Nicotiana attenuata LOX3	AAP83138	629/630	Cys/Phe
Solanum tuberosum H3	CAA65269	631/632	Cys/Phe
Solanum tuberosum H1	CAA65268	614/615	Ser/Phe
Solanum lycopersicum LOXC	AAB65766	611/612	Ser/Phe
Solanum lycopersicum LOXD	AAB65767	625/626	Cys/Phe

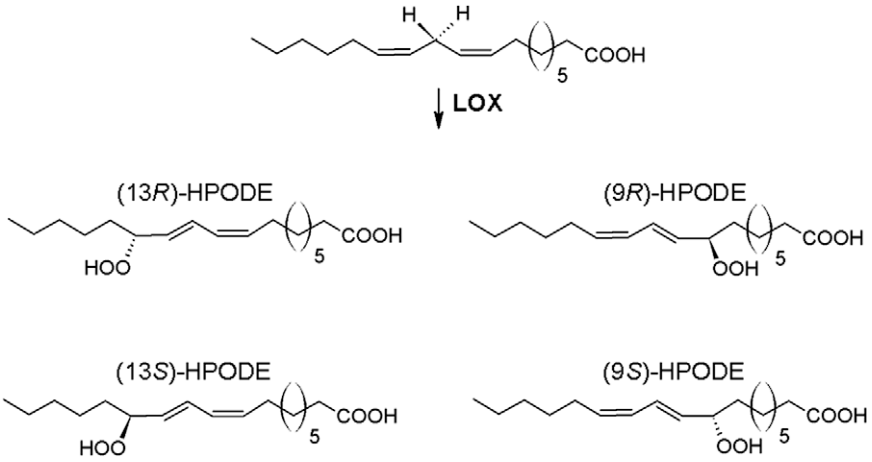

Fig. 5. The LOX reaction and the stereochemistry of the reaction mechanism.

(Liavonchanka and Feussner, 2006). According to this model, a substrate fatty acid may penetrate the active site with its methyl end ahead, leading to linoleate 13-lipoxygenation (Fig. 4, upper panel). Alternatively, the substrate may enter the active site in an inverse "head-to-tail" orientation and linoleate 9-lipoxygenation becomes plausible (Gardner, 1989) (Fig. 4, lower panel).

At first, concepts explaining this regio-specificity of the LOX reaction were developed on the basis of analyzing the stereochemistry of hydrogen abstraction and oxygen insertion (Egmond et al., 1972; Gardner, 1989; Veldink et al., 1972). Later, structural data and models of enzyme substrate interaction were used (Browner et al., 1998). For plant LOXs they were first substantiated by structural modelling and site-directed mutagenesis data for the lipid body 13-LOX from cucumber (Hornung et al., 1999). At the bottom of the substrate-binding pocket, a space-filling histidine or phenylalanine residue was identified, which occurs in nearly all plant 13-LOXs (Table 1), but was first identified for reticulocyte LOX from humans by Sloane et al. (1991). In contrast, for all plant 9-LOXs a small valine residue was identified at this amino acid position (Hornung et al., 1999). In the lipid body 13-LOX, this histidine in the substrate-binding pocket was identified as the primary determinant for positional specificity and its replacement by less space filling residues, such as valine or methionine, altered the positional specificity of a linoleate 13-LOX into a 9-LOX. Structural modelling of the enzyme/substrate interaction suggested that this mutation may de-mask a positively charged guanidino group of an arginine residue at the bottom of the substrate pocket. This guanidino group may then be able to form a salt bridge with the carboxylic group of the substrate, favouring an inverse head-to-tail substrate orientation (Hornung et al., 1999). This special role of this arginine residue may also be supported by the fact that it is highly conserved in plant LOXs. Together, the space within the active site and the orientation of the substrate are both important determinants for the positional specificity of plant LOXs, and are modified by additional factors such as substrate concentration (Kühn et al., 1990), the physico-chemical state of the substrate (Began et al., 1999), pH (Gardner, 1989), or temperature (Georgalaki et al., 1998). Recently, the orientation dependent model has been applied to analyze the substrate orientation for LOX1 from soybean again (Ruddat et al., 2004). Although the enzyme is a 13-LOX at alkaline pH, the substrate seems to bind in an inverse orientation with its carboxylate group within the active site. Moreover evidence is provided that the carboxylate group forms a salt bridge to arginine 707 that was first identified in the lipid body LOX (Hornung et al., 1999). In addition, a LOX isoform from *Mormordica charantia* seeds that carries a glutamine at the position where other plant LOXs harbour either histidine/phenylalanine (linoleate 13-LOX) or valine (linoleate 9-LOX) residues (Hornung et al., 2008) was cloned and characterized. Analyzing the reaction selectivity of this enzyme it turned out that it exhibits a variable positional specificity of LA oxygenation depending on the pH of the reaction mixture. Below pH 7.5 the enzyme showed 9-LOX activity, whereas at higher pH 13-LOX activity was dominant.

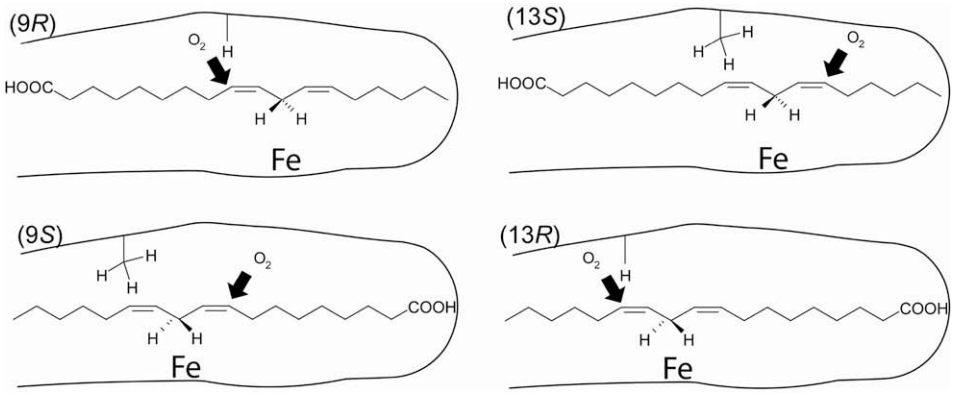

Fig. 6. Active site model for stereo-specificity of the LOX reaction applying the model of inverse substrate orientation. In case of a tail-first orientation in the active site and the reaction begins with an abstraction of D-hydrogen. For *S*-LOXs oxygen attack takes place on the other side of the molecule, because the bulky Ala residue in the "Coffa site" allows oxygen insertion at the C-9 of the fatty acid. Is the Ala replaced by a less bulky Gly the substrate binding takes place in the same orientation, but oxygen attack and results in C-13 "*R*"-oxygenation and vice versa.

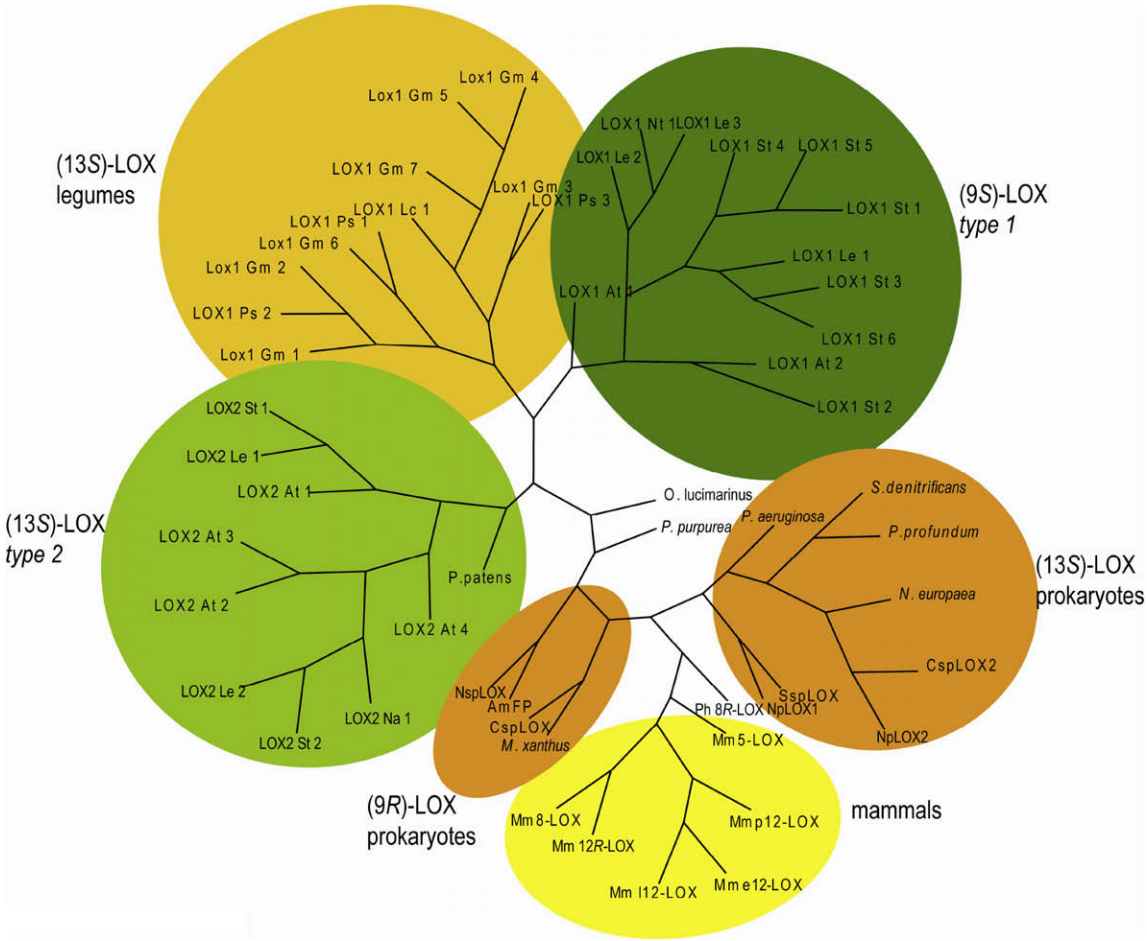

Fig. 7. Phylogenetic analysis of LOX proteins from plants, mammals, moss, corals, algae and cyanobacteria. The circles indicate the grouping of the enzymes into subcategories. The analysis was performed with Phylip3.5 (Department of Genome Sciences, University of Washington) (Felsenstein and Churchill, 1996) using default parameters. In short, alignments were calculated with ClustalX and the analysis was performed with Phylip3.5 using a 100 bootstrap dataset. The tree was constructed with TreeView. The proteins mentioned in the tree refer to the corresponding accession numbers in the gene bank. For clarification within the tree only sequences from distinct plant species have been included and have been partially renamed according to Shibata et al. (1994). *Arabidopsis thaliana*: LOX1:At:1 (AtLOX1, Q06327), LOX2:At:1 (AtLOX2, P38418), LOX2:At:2 (AtLOX3, AAF79461), LOX2:At:3 (AtLOX4, CAC19364), LOX1:At:2 (AtLOX5, CAC19365), LOX2:At:4 (AtLOX6, CAG38328); *Glycine max*: LOX1:Gm:1 (soybean LOX1, AAA33986), LOX1:Gm:2 (soybean LOX2, AAA33987), LOX1:Gm:3 (soybean LOX3, CAA31664), LOX1:Gm:4 (soybean vlxa, BAA03101), LOX1:Gm:5 (soybean vlxb, AAB67732), LOX1:Gm:6 (soybean vlxc, AAA96817), LOX1:Gm:7 (soybean vlxd, S13381); *Solanum lycopersicum*: LOX1:Le:1 (tom-LOXA, P38415), LOX1:Le:2 (tomLOXB, P38416), LOX1:Le:3 (tomLOXE, AAG21691), LOX2:Le:1 (tomLOXC, AAB65766), LOX2:Le:2 (tomLOXD, AAB65767); *Nicotiana tabacum*: LOX1:Nt:1 (NtLOX, S57964); *Nicotiana attenuata*: LOX2:Na:2 (NaLOX3, AAP83138); *Pisum sativum*: LOX1:Ps:1 (AAB71759), LOX1:Ps:2 (CAA55318), LOX1:Ps:3 (CAA55319); *Solanum tuberosum*: LOX1:St:1 (SOLTULOX1, S44940), LOX1:St:2 (STLOX, AAD09202), LOX1:St:3 (StLOX1, P37831), LOX1:St:4 (CAA64766), LOX1:St:5 (CAA64765), LOX1:St:6 (POTLX-2, AAB67860), LOX2:St:1 (StLOXH1, CAA65268), LOX2:St:2 (St-LOXH3, CAA65269); *Lens culinaris*: LOX1:Lc:1: (LLOX, CAA50483); *Mus musculus*: Mm 5-LOX (AAC37673), Mm 8-LOX (CAA75003), Mm 12*R*-LOX (CAA74714), Mm p12-LOX (AAA20659), Mm e12-LOX (CAA67625.1), Mm l12-LOX (AAA20658); *Physcomitrella patens* (PpLOX1, CAE47464); *Porphyra purpurea* (LOX Ppu1, AAA61791); *Pseudomonas aeruginosa* (Q8RNT4); *Nitrosomonas europaea* (NP_841292); *Shewanella denitrificans* OS-217 (YP_562687); *Photobacterium profundum* 3TCK (ZP_01218321); *Myxococcus xanthus* (YP_629995); *Nostoc punctiforme*: NpLOX1 (ZP_00106490), NpLOX2 (ZP_00107030); *Nostoc* sp. (NP_478445); *Plexaura homomalla*: Ph 8*R*-LOX (O16025); *Acaryochloris marina*: AmFP (YP_001516910); *Ostreococcus lucimarinus* (ABO97194.1); *Cyanothece* sp.: CspLOX1 (ZP_02940504), CspLOX2 (ZP_03142690); *Synechococcus* sp.: SspLOX (EDX83314).

In addition, it should be noted that LOXs have been considered for a long time to oxygenate mainly free PUFAs, but a number of studies now provide evidence that at least certain LOXs oxygenate also esterified fatty acid substrates, such as phospholipids or galactolipids (Brash, 1999; Maccarrone et al., 1994; Murray and Brash, 1988; Perez-Gilabert et al., 1998), triacylglycerols (Feussner et al., 1997, 1998; Fuller et al., 2001; Gerhardt et al., 2005; Holtman et al., 1997) or cholesterol esters (Belkner et al., 1991, 1998). All these studies taken together suggest that the fatty acid alignment at the active site appears to be crucial for the understanding of the regiochemistry. However, most concepts do not consider the fact that this alignment is a dynamic process, fatty acid substrates are flexible molecules and molecular dynamics simulations are necessary to visualize this high degree of motional flexibility. Fatty acid substrates may adopt several thermodynamically favoured configurations but only one of these conformers may constitute the catalytically productive conformation. So far there are almost no experimental data characterizing the degree of motional flexibility of the substrates or their reaction intermediates, when bound at the active site of the enzyme. Small angle X-ray scattering suggested a higher degree of motional flexibility of the rabbit enzyme when compared with the soybean LOX1 (Dainese et al., 2005; Hammel et al., 2004). More recent comparative fluorescence life time measurements on the two enzymes confirmed these findings and also provided evidence that suggested a higher degree of motional flexibility of the substrate inside the active site (Mei et al., 2008). For interpretation of future experiments dynamic features of protein substrate interaction should increasingly been considered.

2.2. Stereo-specificity

While the regio-specificity of LOXs has been the focus of a number of studies, recent publications deal with the mechanism of stereo-specificity control of the LOX reaction. An overview over the four potential isomers and enantiomers in case when LA is the substrate is shown in Fig. 5. Interestingly, only a single conserved amino acid in the active site of the enzyme seems to be involved in this part of the reaction in all LOXs analyzed so far (Coffa et al., 2005). The so-called "Coffa site" is reported to be a conserved alanine residue in *S*-specific LOXs and a glycine in all *R*-LOXs. Mutational studies converting the glycine to an alanine in enzymes of the latter category succeeded in partially switching the position of oxygenation and chirality of the product, thus converting a 13*S*- into a 9*R*-LOX enzyme (Coffa et al., 2005). This can be explained again by the model of inverse substrate orientation as shown in Fig. 6 and the assumption that in all enzyme variants oxygen is introduced from the same side. In case of 13*S*- and 9*R*-LOX enzymes the substrate penetrates the substrate binding pocket with its methyl end first (Fig. 6, upper panel). While in a 13*S*-LOX the C-9 position is shielded by the methyl group of an alanine residue, this side chain is missing in a 9*R*-LOX (alanine/glycine exchange) resulting in the preferred dioxygenation at C-9. In case of 9*S*- and 13*R*-LOX enzymes the substrate aligns in a "head-to-tail" orientation (Fig. 6, lower panel) and here the C-13 position is shielded by the methyl group of an alanine residue resulting in 9*S*-LOX activity. Notably, this model has few exceptions. Among *S*-enzymes such an exception is the mouse platelet-type 12*S*-LOX, which has a serine in this position (Coffa and Brash, 2004). Recently, from the cyanobacterium *Nostoc* (*Anabaena*) sp. PCC 7120 a LOX belonging to a separate class of catalase–LOX fusion proteins with high homology to the characterized AOS–LOX fusion protein from the coral *Plexaura homomalla* was isolated (Koljak et al., 1997; Lang et al., 2008; Schneider et al., 2007a). The carboxy-terminal domain of this enzyme was shown to be a fully functional LOX. Sequence alignment of the protein revealed that an alanine aligned with the "Coffa site" suggesting that the protein would be a 13*S*-LOX, but it converted LA into (9*R*)-HPODE (Lang et al., 2008; Zheng et al., 2008). However, when the alanine residue was replaced by a bulkier isoleucine residue, analysis of the reaction products showed indeed that this mutant produced almost exclusively 13*S*-HPODE. More detailed analysis revealed that the substrate orientation was not altered and from sequence alignments with other 13-LOXs it seems very likely that this unique (9*R*)-LOX has evolved from a prokaryotic (13*S*)-LOX by a deletion of one or more sequences leading to an altered active site geometry (Andreou et al., 2008; Lang et al., 2008; Zheng et al., 2008).

3. Perspectives

The latest genomic advances will certainly stimulate the field of oxylipin enzymology and in Fig. 7 a phylogenetic tree analysis is shown where some of the known plant and animal LOX enzymes are compared with new and mainly putative LOX forms from prokaryotes. The isolation and characterization of these new oxylipin forming enzymes will provide further insights into the reaction mechanism of LOXs and their contribution to formation of little-known or even new oxylipin species with yet unknown functions in cellular development and stress response.

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